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Phylogenetic comparison of Coat Protein nucleotide and amino acid sequence of *Cucumber green mottle mosaic virus* from Khorasan province with other isolates of the world

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Abstract

Cucumber green mottle mosaic virus (CGMMV) infects many cucurbit species in the world and causing mottle and systemic mosaic symptoms on cucurbitaceous plant leaves. During a survey, Out of 198 samples of cucurbit plants that were collected from the different fields and cucumber greenhouses in khorasan province, 39 samples were infected by CGMMV in DAS-ELISA using specific polyclonal antibody (The rate of infection was 19.7%). Using specific primers which were designed based on the CGMMV coat protein (CP) gene sequence; a fragment with 486bp length was amplified by reverse transcription polymerase chain reaction (RT-PCR). The CP gene was cloned, sequenced and compared with the sequence of homologous gene of other CGMMV isolates recorded in GeneBank. Phylogenetic analysis using 486 nucleotide long sequences of coat protein gene showed that all CGMMV sequences can be placed into two groups: I and II. Members of group I were divided into two subgroups: A, B. Iranian isolate (kalat) was classified in the group IA. The result showed it had high homology with the other isolates (the highest homology could reach 97.9% in nucleic acid level). As well, phylogenetic tree based on CP amino acid sequences was established and the highest amino acid homology was 98.1%. This is the first report of CP sequence of CGMMV Iranian isolate, determination of its phylogenetic relationship and comparison of its nucleotide and amino acid sequences with those of the other isolates of CGMMV obtained from GenBank.

Key words: *Cucumber green mottle mosaic virus***,** cloning, coat protein sequence, phylogeny

Introduction

Virus infections are a major limiting factor in cucurbit production in Iran. *Cucumber green mottle mosaic virus* belongs to the genus *Tobamovirus* and the family *Virgaviridae* that infects many cucurbit species in the world and causing mottle and systemic mosaic symptoms on cucurbitaceous plant leaves (Kim *et al*.,

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2003). Tobamoviruses are readily spread mechanically within the field by handling and mechanical damage to plants, but not by insect or by fungal vectors (Choi *et al*., 2002). The nucleocapsid of CGMMV has a rod shape with dimensions nearly of 300-320 ×18 nm; its genome is a positive-sense, single-stranded RNA of around 6450-nt long which contains four open-reading frames (ORFs) encoding four proteins: two replicas proteins (130 and 180 kDa), one movement protein (MP, 30 kDa) and one coat protein (CP, 17 kDa). (Tan *et al*., 2000; Lewandowski, 2000) rod-shaped particles of virus are very stable and simple. Virus capsid is not enveloped. CGMMV was first described in cucumber, and named cucumber virus 3 (CV3) and cucumber virus 4 (CV4) by Ainsworth in 1935 (Ugaki *et al*., 1991). Several strains of CGMMV have been reported from Europe, Israel, Korea, Pakistan, India and Japan (Lee, 1996). Most strains have a narrow host range limited to family *Cucurbitaceae*. (Antignus *et al*., 2001). The *Cucurbitaceae* family includes several species of horticultural crops that have great economical important like watermelon, cucumber, squash and melon. Iran cultivates the second-largest total cucurbit acreage (770,000 ha) in the world, exceeded only by China, and is the third-largest producer of cucurbits after China and Turkey. Cucurbits in greenhouses or under plastic covers are grown in most areas of Iran, with cucumber accounting for approximately 98% and watermelon and melon for 1.5% and 0.5% of the total acreage, respectively (Massumi *et al*., 2007). Among the Cucurbitaceous crops cucumber is sensitive to many viruses and shows a more severe reaction to viruses (Lovisolo, 1980).

Although CGMMV is widespread in the world, there are few reports on its occurrence in Iran. CGMMV was reported in Markazi province by Ghorbani in 1986 (Safaeizadeh, 2008), it has not been reported in khorasan province (North-East of Iran) until now; also a report from genetic sequence of CGMMV isolates has not yet been published in Iran. ELISA and RT-PCR are methods commonly used to detect CGMMV (Liu *et al*., 2009). In addition, several molecular techniques have been widely used for the diagnosis of plant viruses, allowing the detection of very small amounts of a virus, and also the cloning of genomic fragments of viruses (Henson and French, 1993). The aims of this study were to study of the existence and spread of CGMMV in cucurbitaceous growing areas in North-East of Iran and to identify CGMMV at the molecular level.

Materials and Methods

Virus sources

Samples were collected from June 2009 to October 2010 from different cucurbit plants including cucumber, bottle gourd, zucchini squash, watermelon (*Citrullus lanatus*) and melon (*Cucumis melo* varieties including Cantaloupe, snakemelon, longmelon and local cvs.) from fields and cucumber greenhouses in major cucurbit growing areas of khorasan province (Torbat-e-Jam, Neyshabour, Mashhad, Chenaran, kashmar, kalat, Sabzevar) in Iran. Samples (n=198) consisted of young fully expanded leaves showing Symptoms such as mottling and systemic mosaic, mild to severe yellow mottling, vein clearing and blistering.

Double-antibody Sandwich (DAS)-ELISA

DAS-ELISA was performed on Cucurbit leaf samples following the general protocol of Clark and Adams (1977). The commercial antiserum (SEDIAG S.A,

Strasbourg, France) and conjugate were used at 1:200 dilutions. Wells of micro titer Plates were coated with 100 µl of each immunoglobulin G in carbonate coating buffer (0.06 M Na_2CO_3 and 0.14 M $NaHCO_3$, pH 9.6) and incubated at 37°C for 4 h ; the assays were carried out according to the manufacturer's materials and methods. Extracts from healthy *C. sativus* were used as negative control in all tests. Reactions were measured spectrophotometrically at 405 nm using an ELISA plate reader (STAT FAX 2100, USA). A sample was considered virus-positive if the optical density (OD) exceeded the mean plus three standard deviations of the OD of the healthy controls. A typical CGMMV isolate (CV4) was used as reference virus in the serological, biological and molecular detection testes. Samples that showed high ELISA values were selected for RT-PCR analysis.

Total RNA isolation and RT-PCR

Leaf tissue (100 mg) was ground into fine powder in liquid nitrogen, and then transferred to microtube. Total RNA was extracted from pulverized tissue using AccuZol™ Reagent (Bioneer, Alameda, CA) according to the manufacturer's instructions. After precipitating with ethanol, total RNA was re-solubilized in 25µl of RNase-free water. The complete sequence of the SH strain of CGMMV (CGMMV-SH. GenBank accession number D12505) was used for designing Forward and Reverse primers. Forward primer (5'- ATGGCTTACAATCCGATC AC -3', at Positions 5763-5782) and reverse primer (5'- CTACCACCTCGAAAG CTTAG -3', at Positions 6229-6248) covered a full CP gene. Reverse transcription (RT) reaction was performed as follows: 1µl of reverse primer (20 picomoles) and 1µl of RNA sample were added to 8.5µl of diethyl pyrocarbonate (DEPC)-treated water. The mixture was incubated at 65˚C for 10 min and chilled on ice for 3 min to denature the RNA. Then 3.5µl of DEPC-treated water, 4µl of 5x M-MLV RT buffer, 2µl of dNTPs mix (10 mm) and 1µl M-MLV(200 U µl) reverse transcriptase (Promege, USA) were added to mixture. The RT reactions were incubated at 42˚C for 60 min followed by 95 ˚C for 3 min to terminate the RT reaction. Viral cDNA was then amplified by PCR. The PCR reaction was performed using 2.5µl of cDNA, 13.5µl DEPC-treated water ,5µl of 5x GoTag polymerase buffer, 2.5µl 10x MgCl2, 0.5µl of each forward and reverse primers(20 pmol), 0.75µl of dNTP mix (10mm) and 0.125µl of GoTag polymerase(2.5 U µl) (Promege, USA). Following program was used for PCR: A first denaturation for 3 min at 94 ˚C was performed and followed by 35 cycle of denaturation for 45 s at 94˚C, 30 s of annealing at 60˚C, extension for 1 min at 72˚C, and a final extension step at 72˚C for 7 min. PCR products were analyzed by electrophoresis in 1.2% agarose gel and visualized by ethidium bromide staining.

Cloning and sequencing

RT-PCR products of CGMMV isolates were then purified from 1% agarose gels using the DNA gel extraction kit (bioneer, korea) and cloned into the pDrive vector using InsT/Aclon PCR product cloning kit (Qiagen, USA) according to the manufacturer's instructions, and transformed into *Escherichia Coli* strain DH5α. Plasmid DNA from recombinant clones was purified using the high pure plasmid isolation kit (Roche, Germany) and nucleotide sequencing reactions were performed by MWG Company (Biotech, Germany) using dideoxy nucleotide

chain termination method with M13-forward and reverse primers. This isolate and 19 isolates from other parts of the world present in GenBank were analyzed to determine their phylogenetic relationship.

Phylogenetic analysis

The phylogenetic analysis of the Iranian isolate was conducted by comparing the 486 bp of the CP gene with those of the other isolates of CGMMV obtained from GenBank (Table 1). Nucleotide sequence analysis and translation to the corresponding amino acid sequence were performed using DNAMAN software (version 4.02) package (BBA, Germany). The nucleotide and deduced amino acid sequences were compared with the equivalent sequences of CGMMV. Multiple alignments of the nucleotide and amino acid sequences were carried out using the ClustalW program in BioEdit (version 7.0.9.) software and DNAMAN software (version 4.02). Phylogenetic trees for grouping based on nucleotide and amino acid sequences were constructed by MEGA 4.1 software program using similarity matrix and the neighbor-joining method. Tree branches were bootstrapped with 1000 replications and bootstrap values less than 50 % were condensed on nodes (Figure 2).

Results

Virus isolate

Among 198 samples collected from different areas of Khorasan province, 39 samples were positive in ELISA test (The rate of infection was 19.7%).

RT-PCR, sequence data and identity matrix

In order to provide a fast and sensitive detection method in addition to host reaction and serological tests for the diagnosis of disease caused by CGMMV, a RT-PCR method was developed. The PCR-amplified of approximately 490 bp was obtained (Figure 1a) by using specific primers CGMMV-F and CGMMV-R, when RNA extracted from isolates was used as the template for the first- strand cDNA synthesis. However, some samples were evaluated as positive in ELISA test but no DNA fragments were amplified from total RNAs extracted from this samples. Anyway, the RT-PCR products were cloned into pDrive vector (3.8 kb) (Figure 1b) and one of them sequenced.

Sequence information for kalat isolate has been submitted to NCBI-GenBank with the accession number HQ329106. The identity of CP nucleotide sequence of kalat isolate in comparison with other GenBank isolates of CGMMV ranged from 89 to 98 %. The Iranian isolate in group IA displayed the lowest (88.9%) and the highest (97.9%) nucleotide identity sequence with Ukraine and W-Japan, respectively (Table 2).

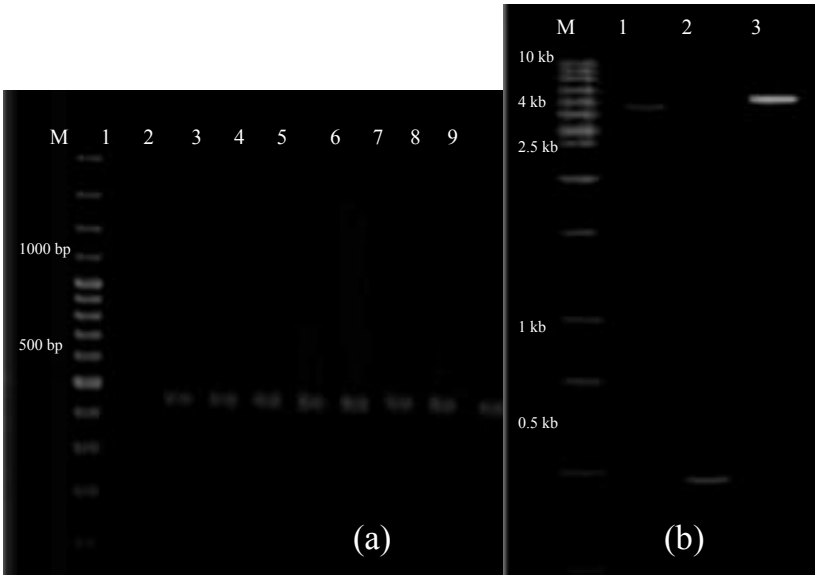

Figure 1. (a) Electrophoresis pattern of DNA fragments amplified by RT-PCR in 1.2 % agarose gel related to seven selected CGMMV isolates collected from different location of Khorasan province (lanes 3-9) 1: healthy cucumber plant extract as negative control. 2: CV4 isolate. M: 100 bp DNA marker (Fermentas) **(b)** M: 1- kb DNA ladder, lanes 1-3, represent pDrive vector (3.85 kb), CP amplified gene of kalat isolate (486 bp), transformed fragment (about 4.25 kb), respectively.

Table 1. GenBank accession numbers and origin of previously reported CGMMV Isolates/strains[a] used for phylogenetic comparison of the 486 nucleotides long fragment of genome including CP gene

Isolate or strain	Accession no.	Host	Origin	Number of nucleotides
CGMMV-W	AB015146	Watermelon	Japan	6424
CGMMV-SH	D12505	Watermelon	Japan	6424
CGMMV-Y	DQ399708	Watermelon	South Korea	524
CGMMV-KOM	AF417243	Oriental melon	South Korea	6424
CGMMV-KW	AF417242	Watermelon	South Korea	6424
CGMMV-W(kor)	AF225984	Watermelon	South Korea	634
CGMMV-GR3	AJ459421	Watermelon	Greece	486
CGMMV-GR7	AJ459423	Watermelon	Greece	486
CGMMV-GX-G	DQ647384	Cucumber	China	654
CGMMV-LHP	DQ997778	Cucurbits seed	China	524
CGMMV- Bottle gourd	DQ767636	Bottle gourd	India: Rajasthan	486
CGMMV-AL1	AJ748352	Bottle gourd	India	486
CGMMV -MC-1	EF521882	Cucumber	Russia	484
CGMMV- MC-2	GQ495274	Cucumber	Russia	6422
CGMMV-pak	AB127937	Bottle gourd	Pakistan	1012
CGMMV-zucchini	AB194531	Zucchini	Indonesia	486
CGMMV -SP	GQ411361	Cucumber	Spain	6422
CGMMV- Ukraine	FJ426406	Cucumber	Ukraine	542
CGMMV-BG	FJ654659	Bottle gourd	Taiwan	6422
CGMMV-kalat	**HQ329106**	**Cucumber**	**Iran**	**486**
KGMMV(out group)[b]	AF239212	Zucchini	Korea	1102

[a]: The CGMMV isolates were identified by location, plant host, and series number
[b]: KGMMV is *Kyuri green mottle mosaic virus* included as outgroup

Phylogenetic analysis

Phylogenetic tree based on multiple sequence alignment of CP divided all CGMMV isolates into two large groups: group I included 2 subgroups A, B (Figure 2a). Isolates in group II included European isolates (Spain, Ukraine,

Greece and Russia). Group I included Asian isolates and GR7 isolate from Greece. Iranian isolate (kalat) with Japan, China, Korea, India, Indonesia, Taiwan isolates were classified in the subgroup IA, which can be divided into many clades and Iranian isolate fall into distinct clade. Only Pakistan isolate was classified in subgroup IB. In phylogenetic analyses based on ClustalW Multiple alignments, the Iranian isolate in group IA displayed the lowest (88.9%) and the highest (97.9%) nucleotide sequence identity with the isolates Ukraine and W-Japan, respectively. Comparison of the CP amino acid sequence showed exiting the lowest (76.4 %) between CGMMV-Kalat and GR3 (Greece) and the highest (98.1%) amino acid homology between CGMMV-Kalat and isolates from AL1 (India), W (korea), GX-G (China), LHP, W (Japan), Zucchini (Indonesia), Kom and KW (south Korea) (Table 2). In phylogenetic tree based on analysis of the CP amino acid sequences the isolates initially were clustered into two groups and then each group was divided into many subgroups (Figure 2b). The percentage similarities for nucleotide and amino acid between kalat isolate and fourteen isolates clustered in group I ranged from 88.9 to 97.9 % and from 76.4 to 98.1, respectively.

Discussion

Many tobamoviruses are distributed worldwide and can infect many field and greenhouse crops (Zhang *et al*., 2008). Cucurbitaceous crops are an important part of diverse nutrition diet worldwide, grown commercially throughout the world, have been destroyed completely locally or epidemically when infected by plant viruses (Yoon *et al*., 2008). CGMMV causes one of the most common diseases in the family *Cucurbitaceae* (Slavokhotova *et al*., 2007).

Results of this study suggest that CGMMV-Kalat isolated from cucumber in Khorasan province has properties common with the genus of *Tobamovirus* (Choi *et al*., 1998). These include serological relationships, RT-PCR and nucleotide sequence of the CP gene. DAS-ELISA is a common diagnostic technique in survey studies to detect plant viruses. However, the RT-PCR technique is highly sensitive, simple and useful in overcoming many difficulties encountered with serological methods. Although all samples showed systemic mosaic and mild to severe yellow mottling symptoms, only 19.7% of samples were infected with CGMMV. This low percentage might be due to the fact that mottle mosaic is a complex disease that can be caused by different viruses such as *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus-2* (WMV-2), *Squash mosaic virus* (SqMV), *Zucchini yellow mosaic virus* (ZYMV), as well as by CGMMV (Shabanian *et al*., 2007) and the symptoms observed may have been caused by one or more of those other viruses.

Phylogenetic analyses were done by the neighbor-joining (NJ) method implemented with ClustalW alignment which was used to compare the similarities between the coding nucleotides, and their encoded protein. Sequencing results of a Coat portion confirmed the RT-PCR analysis and showed slight variations between the sequence of the CP gene of CGMMV and that of other strains from other parts in the world. The percentage identities for nucleotide and amino acid between CGMMV-kalat and fourteen isolates clustered in group-I ranged from 88.9 to 97.9 % and from 76.4 to 98.1, respectively. This confirms that CGMMV-Kalat isolated from cucumber has belonging to genus of *Tobamovirus*.

Table 2. Comparison of the Percent nucleotide (top and right) and amino acid (bottom and left, in bold) identities of 486 nt CP sequences between selected CGMMV isolates[a] used in this study

	1. Kalat	2. Pak	3. AL1	4. Y	5. W (kor)	6. GX-G	7. LHP	8. SH	9. W (Jap)	10. BG	11. Zucchini	12. GR3	13. GR7	14. SP	15. MC-1	16. MC-2	17. Ukraine	18. Bottle-g	19. KW	20. KOM
1	*	97.1	97.7	96.9	97.3	97.3	97.3	97.3	97.9	97.1	97.5	90.9	97.5	90.3	91.3	91.3	88.9	97.7	97.1	97.3
2	97.5	*	98.1	97.3	98.1	97.7	97.7	97.7	98.3	97.5	97.9	91.5	97.7	90.9	91.9	91.9	89.3	97.5	97.5	97.7
3	98.1	99.4	*	98.7	99.1	98.3	99.1	98.9	98.9	98.3	99.3	91.9	99.1	92.3	98.3	92.3	89.7	98.9	98.9	99.1
4	96.9	98.1	98.8	*	99.1	97.9	99.5	99.1	98.5	98.9	99.3	91.7	98.3	90.7	91.7	91.7	89.1	98.1	99.3	99.1
5	98.1	99.4	100	98.8	*	97.9	99.5	99.5	99.5	98.5	99.3	98.5	92.5	98.7	91.5	92.5	89.9	98.5	99.3	99.5
6	98.1	99.4	100	98.8	100	*	98.3	97.9	99.3	97.7	98.1	91.3	97.9	90.7	91.7	91.7	89.1	97.7	98.1	97.9
7	98.1	99.4	100	98.8	100	100	*	99.5	98.9	99.3	99.7	92.1	98.7	91.1	92.1	92.1	89.5	98.5	99.7	99.5
8	98.1	99.4	100	98.8	100	100	100	*	98.5	99.3	99.7	92.1	98.7	91.1	92.1	92.1	89.5	98.5	99.3	99.5
9	98.1	99.4	100	98.8	100	100	100	100	*	98.3	98.7	91.5	98.5	90.9	91.9	91.9	89.3	98.3	98.7	98.3
10	87.0	87.6	88.2	87.0	88.2	88.2	88.2	88.2	88.2	*	99.5	91.9	98.5	90.9	91.9	91.9	89.3	98.3	91.9	98.3
11	98.1	99.4	100	98.8	100	100	100	100	100	88.2	*	92.3	98.9	91.3	92.3	92.3	89.7	98.7	99.5	99.7
12	76.4	77.1	77.7	77.1	77.1	77.1	77.1	77.1	77.1	77.1	77.1	*	92.1	95.8	97.5	97.5	94.4	91.1	92.1	92.3
13	97.5	98.1	98.8	97.5	98.8	98.8	98.8	98.8	98.8	87	98.8	77.1	*	91.5	92.5	92.5	89.9	98.5	98.5	98.7
14	93.8	95.0	95.7	94.4	95.7	95.7	95.7	95.7	95.7	84.5	95.7	.75.8	95	*	98.3	98.3	95.6	90.5	91.1	91.3
15	97.5	98.8	99.4	98.1	99.4	99.4	99.4	99.4	99.4	87.6	99.4	78.3	98.8	96.3	*	100	96.7	91.5	92.1	92.3
16	97.5	98.8	99.4	98.1	99.4	99.4	99.4	99.4	99.4	87.6	99.4	78.3	98.8	96.3	100	*	96.7	91.5	92.1	92.3
17	82.4	83.0	83.6	82.4	83.6	83.6	83.6	83.6	83.6	81.2	82.5	87.8	81.2	88.5	84.3	84.3	*	88.9	89.5	89.7
18	97.5	98.1	98.8	97.5	98.8	98.8	98.8	98.8	98.8	87	98.8	76.4	97.5	94.4	98.1	98.1	82.4	*	98.3	98.5
19	98.1	99.4	100	98.6	100	100	100	100	100	88.2	100	77.7	98.8	95.7	99.4	99.4	83.6	98.8	*	99.3
20	98.1	99.4	100	98.6	100	100	100	100	100	88.2	100	77.7	98.8	95.7	99.4	99.4	83.6	98.8	100	*

[a]: Isolates and Strains are designated as 1: Kalat (Cucumber: Iran), 2: Pak (pakistan), 3: AL1 (Bottle gourd: India), 4: Y (South Korea) 5: W (watermelon: korea), 6: GX-G (Cucumber :China), 7: LHP (Cucurbits: China), 8: SH (watermelon: Japan), 9: W (watermelon: Japan), 10: BG (Bottle gourd :Taiwan), 11: Zucchini(Indonesia), 12: GR3(watermelon :Greece), 13: GR7(watermelon :Greece), 14: SP(Cucumber :Spain), 15: MC-1(Cucumber: Russia), 16: MC-2(Cucumber: Russia), 17: Ukraine(Cucumber: Ukraine), 18: Bottle–g (Bottle gourd: India), 19: KW(Watermelon: Korea), 20: KOM(Oriental melon: Korea).

The research provides clear evidence that CGMMV occurs in Khorasan province. However, from an epidemiological point of view; much work still needs to be done. For example, the source of CGMMV must be investigated and its spread to locations where it does not occur should be prevented. Here, it is important to note that, due to the lack of efficient products for chemical treatment under field conditions, control of diseases caused by viruses is difficult. Consequently, preventive measures to avoid planting of contaminated material are of the highest importance in the context of contaminated material of an integrated approach to control. Tobamoviruses form very stable particles making them easy to spread and difficult to eliminate. So the best defense against tobamoviruses is avoidance and the first line of defense is to grow plants with resistance to tobamoviruses. The main danger for the cucumber plants is free virus, which is released into the soil after decay of the plant debris (Budzanivska *et al*., 2006). Virus-free seeds should be used and care should be taken to not infect transplants during production to ensure that only uninfected transplants are set in the field. As the virus is seed transmitted and easily transmitted mechanically (Hull, 2002) efforts have been made to produce virus-free seeds. Hot water and hot air treatments, using widely varying temperature and treatment times, have

demonstrate the most functional methods for inactivating viruses on seeds or plants (Kim and Lee, 2000).

Data shown in this study represent the first report on the characterization of CGMMV at the molecular level in Iran. Previous studies reported the occurrence of the virus (Ghorbani, 1986) but did not provide information about it at the molecular level. Sequencing results of a Coat portion confirmed the RT-PCR analysis and showed slight variations between the sequence of the CP gene of CGMMV and that of other strains from other parts in the world. The present study reports similarity and phylogenetic relationship of CGMMV-kalat from Iran to other isolates of CGMMV recorded in NCBI. The CP gene of CGMMV-Kalat shared 97.9% identity in nucleotide level and 98.1% in amino acid level with W-Japan isolate.

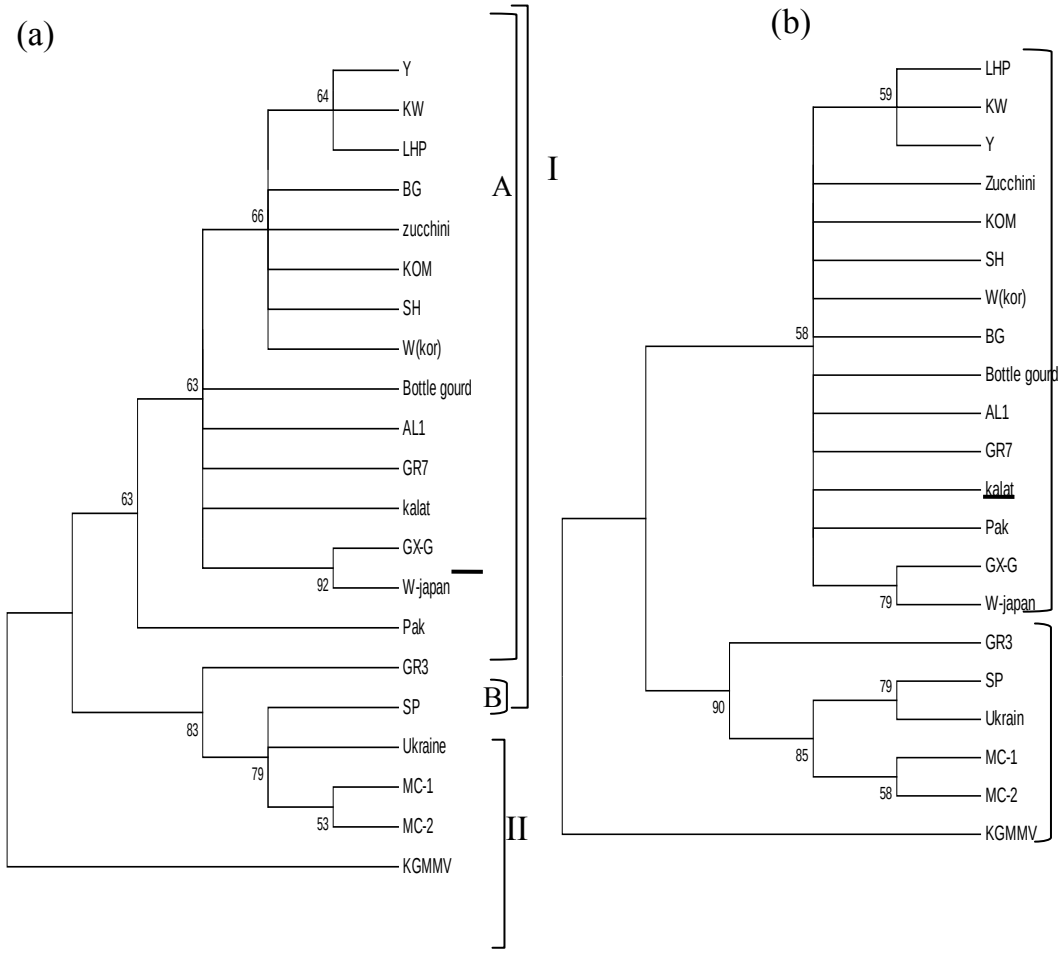

Figure 2. Phylogenetic tree constructed from the alignment of nucleotide **(a)** and amino acid **(b)** sequences of coat protein gene of 20 CGMMV isolates (Table 1) using neighbor -joining method based on 1000 replicates. The numbers indicate bootstrap percentage. Bootstrap values higher than 50 are indicated on nodes, and the nodes less than 50 were condensed. The isolate, which has been sequenced here, was underlined. KGMMV (*Kyuri green mottle mosaic virus*) included as out-group

To our knowledge, this is the first report on the occurrence of CGMMV in cucumber in Iran based on RT-PCR, and CP gene analyses. The data obtained in

this study will be beneficial to improve control strategies for this virus in Iran. Hence, identification and differentiation of isolates and strains of CGMMV, particularly analysis of its genetic diversity and evolution can be finding procedures for evolution and development of the pathogen and as result presenting the appropriate solution to its control.

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